Review Article

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Prenatal Diagnosis of Down Syndrome in a Molecular Era

Ravindra Kumar*, Pratibha Singh**

Abstract

Down syndrome (trisomy 21) is considered to be the most frequent etiology of mental retardation and it is the predominant reason for women seeking prenatal diagnosis. The current gold standard for diagnosis of trisomy 21 is provided by invasive sampling of fetal genetic material through chorionic villus sampling (CVS) or amniocentesis followed by conventional cytogenetic. Now in present molecular era various molecular techniques have been developed for rapid diagnosis of Down syndrome. In review the principles, pros and cons of various techniques involved in invasive and noninvasive prenatal diagnosis of Down syndrome are discussed.

Keywords: Down syndrome; Trisomy 21; Prenatal diagnosis.

Review

Aneuploidy is the most common chromosomal aberration with clinical importance in the human being which exists in 3 to 4% of recognized pregnancies. Probability of occurrence these abnormalities increases with mother's age.[1,2] Down syndrome, the most common fetal aneuploidy, is caused by an extra copy of chromosome 21 affecting 1 in 700-1000 live births.[3]

Identifying the cause of fetal genetic disorders started in the early 1970s. Since its inception, the most common reason for prenatal diagnosis is increased risk of having a child with Down syndrome. This risk is dependent on maternal age, and is also assessed by maternal serum screening and fetal ultrasonography (nuchal translucency), additional structural fetal abnormalities detected by ultrasonography or a previous child with a chromosome disorder.[4-6]

Screening is rational only when a large proportion of the population is tested, which means that a large number of women will present with increased risk of the fetal disorder. This calls for a diagnostic test. Traditionally, fetal diagnosis by Karyotyping most often by Giemsa banding is carried out at the metaphase stage of the cell cycle, when chromosomes are optimally condensed. Amniotic fluid samples obtained by amniocentesis do not contain any fetal cells in division and have to be grown in vitro to obtain cells at the metaphase stage. In chorionic villus samples (CVSs), some fetal cells are spontaneously dividing and cells at metaphase can be used. However, the resolution (of special importance for the detection of structural abnormalities) may be quite low, as these spontaneously dividing cells have more condensed chromosomes than those obtained after cell culture in vitro. Another disadvantage of CVSs is the common occurrence of confined placental mosaicism,

Author's Affiliation: *Scientist and In charge, Central Research Lab, Sri Aurobindo Medical College and PG Institute, Indore, Madhya Pradesh, Pin 453555, **Additional Professor and Head, Obstetrics & Gynecology, All India Institute of Medical Sciences, Jodhpur-342005, Rajasthan, India.

Corresponding Author: Dr Ravindra Kumar, Ph.D. (Genetics) Scientist and Incharge, Central Research Laboratory, Sri Aurobindo Medical College and PG Institute, Indore, Madhya Pradesh, Pin 453555, India.

E-mail ravindrachhabra@gmail.com

the occurrence of a proportion of aberrant cells that do not represent the chromosome status of the fetus. These aberrant cells when originating in cytotrophoblasts may be selected against during cell culture in vitro. Therefore, cell culture is usually performed as a follow-up to any direct chromosome diagnosis on CVSs. However, it is important to note that even at high resolution G-banding, deletions or duplications <5 Mb will usually remain undetected. The implication is that a proportion of chromosome abnormalities, which may be associated with physical and mental disability, will not be routinely diagnosed, even with this 'gold standard' test.

To obtain enough cells in division for karyotyping it takes 2-3 weeks' time and a larger amount of amniotic fluid. It is recognized that long waiting times for results may cause much psychological suffering and this has been one of the main reasons for the introduction of molecular methods for prenatal diagnosis of common chromosome disorders. The smaller amount of fetal tissue may reduce the incidence of abortion that occurs following these invasive procedures. This type of approach does not require cell culture and reports can routinely be issued within 1–2 days. Here author has tried to reviewed the pros and cons of the various molecular techniques used in the prenatal diagnosis of Down syndrome.

Fluorescent in Situ Hybridization (FISH)

FISH is a technique that essentially consists in hybridizing a DNA probe to its complementary sequence on chromosomal preparations previously fixed on slides. Probes are labeled either directly, by incorporation of fluorescent nucleotides, or indirectly by incorporation of reporter molecules that are subsequently detected by fluorescent antibodies or other affinity molecules. Probes and targets are finally visualized in situ by microscopy analysis.[7] As a combined molecular and cytological approach, the major advantage of this technique resides in its unique ability to provide an intermediate degree of resolution between DNA analysis and chromosomal investigations while retaining information at the single cell level.

Ward *et al* had shown accuracy of prenatal detection of the most common aneuploidy by FISH by analyzing 4500 samples.[8] FISH takes only 24-48 hr, costs about half of the conventional cytogenetic technique, and offers an opportunity to reduce anxiety through early decision making process.

The main limitation of FISH, and the factor that prevents cost from being minimized, is its unsuitability for automation.[9,10] Analysis involves considerable time with a skilled technician. Technical artifacts sometimes may cause misinterpretation of the results. The most common artifacts are overlapping or splitting signals. Overlapping signals are usually the result of overlapping chromosomes. Splitting signals are either inherent to a specific probe or owing to separation of sister chromatids. Suboptimal denaturation or hybridization, and inappropriate wash conditions may cause high background or weak signals. Maternal cell contamination can also interfere with interpretation. The addition of FISH to full karyotype approximately doubles the cost to for both tests.[11]

Quantitative Fluorescent Polymerase Chain Reaction (QF PCR)

The development of PCR technology and human genome mapping are land mark contributions that have facilitated the initiation of an alternative approach for the detection of trisomy 21. It involves PCR amplification of polymorphic small tandem repeat (STR) markers located on human chromosome 21 and analysis by fluorescence based method to identify the presence of an additional allele on the third copy of the chromosome. By means of fluorescent primers, the amplified segments can be visualized and quantified as peak areas on automated DNA scanners. Normal heterozygous subjects are expected to show two peak areas (peaks ratio 1:1) for each chromosome analysed, while trisomies are visualized either as an extra peak (triallelic subjects) or as a 2:1 ratio peak between the

two areas. This DNA approach was first used for the diagnosis of the X-chromosome aneuploidy[12] and later it was modified by Mansfield to detect trisomy 21 and 18[13]. QF-PCR is routinely used in the UK and several European countries for detection of the common autosomal aneuploidies and sex chromosome aneuploidies[14-17].

QF-PCR might play a major role and be considered a valid alternative to the full karyotype. Being less expensive, and almost entirely automated, more women could undergo invasive prenatal diagnosis without significant increase in health expenditure. The main advantages of the prenatal diagnosis of trisomy 21 on DNA extracted from uncultured samples of amniotic fluid are: the small amount of fluid required and the speed with which the test can be done. The technique can be automated and 96 samples processed simultaneously, and results are available on the same day.

QF-PCR has some advantages over FISH as QF-PCR can detect maternal cell contamination which cannot be disclosed by FISH in cases of female fetuses. The major drawback here is the need for an informative polymorphism, as even when using multiple sequences, in some patients no informative sequences may be found. The polymorphic loci show different frequencies in different populations, so polymorphisms found to be optimal in one population may be ineffective in another.

Multiple Ligation Probe Amplification (MLPA)

Multiplex quantitative fluorescence PCR (QF-PCR) provides the possibility to detect copy number variation of chromosomal sequences in several hours. It also has the advantage of being much cheaper and allowing the simultaneous processing of larger numbers of samples than FISH and karyotyping analysis. However, the presence of multiple primer pairs in a PCR reaction reduces the reliability of the quantification. To solve these technical problems, multiplex ligation-dependent probe amplification (MLPA) has emerged as an alternative to standard PCR-based techniques for detection of the chromosome aneuploidies.[18] By using identical primers, the reaction efficiencies are equal for each target. In this approach, multiple tagged oligonucleotide probe pairs are hybridized to distinct genomic regions, followed by mismatch--sensitive ligation of only correctly hybridized probe pairs. Then the templates are amplified with common fluorescent primers, the products undergo capillary electrophoresis and the fluorescent peaks are used for quantification. A relative probe signal is calculated for every probe (length) by dividing each individual peak area by the sum of the peak areas of all the probes in the sample and expressed as a multiple of the relative probe signal obtained in a control sample. The expected value for a euploid sample is close to 1 and for a trisomic sample is close to 1.5.

It allows for relative quantification of up to 50 different target sequences in one reaction and does not require living cells or cell culture. It is less labor-intensive and less expensive compared to karyotyping and FISH. Therefore, MLPA has been widely applied for molecular diagnosis of chromosomal aneuploidies.[19, 20] Moreover, a commercial MLPA kit based on length discrimination of the ligation products for detection of aneuploidies on chromosomes 13, 18, 21, X and Y has been developed. Its turn-around time can be as rapid as 30 hours.

The major disadvantages of the MLPA are the inability to detect all cases of triploidy, unknown sensitivity for mosaicism, and assays can be time-consuming and difficult to develop. MLPA is still under investigation and not yet ready for widespread introduction into prenatal diagnosis.

Real Time PCR

Real-time PCR is a system that detects the increments of an amplicon based on fluorescence while the PCR cycle is going on. The amount of target DNA in a sample is indicated by the number of cycles needed to

amplify the target to a detectable fluorescent level, not by the intensity of the fluorescence. By using this procedure it becomes possible to distinguish small differences of the amount of target DNA in a few hours.[21]

Theoretically, the amount of chromosomal genomic DNA on aneuploidy chromosome is 1.5 times higher than those on other chromosomes. If two appropriate genomic fragments located on different chromosomes have similar efficiencies of amplification in a real-time PCR assay, the amounts of genes on respective chromosomes can be compared, which enables rapid diagnosis of aneuploidy. [22-24]

Compared with currently used aneuploidy rapid detection method, fluorescence in-situ hybridization (FISH) and quantitative fluorescent PCR analysis of short tandem repeats, this real-time PCR method is faster and simpler, and do not need post-amplification handling, which leads to high throughput performance and is less susceptible to contamination. Further, as real-time PCR targets are nonpolymorphic sequences, valid results can be generated regardless of the patient population involved. The technique is amenable to automation and high throughput, and the sample demand is little. Further real time PCR machine is much cheaper than DNA sequencer which is necessary in case of both MLPA and QF PCR.

Non Invasive Prenatal Diagnosis of Down Syndrome

Since 1997, when cell free fetal DNA in maternal circulation was discovered, the research interest has focused on the development of reliable techniques for non-invasive prenatal diagnosis (NIPD) that would allow the direct analysis of fetal genetic material based on the discovery of cell-free fetal DNA and RNA in the maternal circulation. The medical significance of the development of NIPD is of great importance as it can potentially be offered to all pregnancies, presents no risk of loss of the pregnancy and provides a more effective prenatal diagnosis compared to currently used invasive methods. The direct analysis of circulating fetal DNA for the NIPD of chromosomal aneuploidies is mainly complicated by the presence of the coexisting background maternal DNA.

Digital PCR

It is a highly sensitive technique which uses dilution to isolate single template DNA molecules to be amplified, in order to detect very small differences in chromosome ratios. [25] Importantly, in this method, fetally derived nucleic acid is not specifically distinguished from maternal DNA; instead, the technique provides a measure of the total (i.e. fetal plus maternal) dosage of a particular chromosome relative to another reference chromosome. Although this technique requires prior enrichment of the cell free fetal DNA to achieve high accuracy, it should be applicable to all cases because it is not reliant on heterozygous SNPs.

Next Generation Sequencing

This method is achieved by high throughput shotgun sequencing of DNA from plasma of maternal peripheral blood, followed by ratio analysis of each chromosome sequence tag density over the median tag density of all autosomes using a z-score analysis.[26,27] The method was further validated using 753 plasma samples of which 86 were from Down syndrome pregnancies and provided 100% and 97.9%, sensitivity and specificity, respectively.[28] Recently, an additional validation study using next generation sequencing was carried out in 1696 cases and demonstrated 98.6% and 99.8%, sensitivity and specificity, respectively.[29]

MeDIP and Real Time qPCR

The most promising demonstrations of NIPD were based on the use of differentially methylated markers (DMRs), which have differences in their methylation status between

fetal DNA and maternal DNA.[30,31] The identification of new DMRs on chromosomes 13, 18, 21, X and Y was achieved by using a method consisting of methylated DNA immunoprecipitation with high resolution oligo-arrays (MeDiP on a Chip).[32] In principle, DNA from non-pregnant female blood and 1st and 3rd trimester placentas were immunoprecipitated in two separate experiments, using a specific antibody for CG-methyl sites. The isolated hypermethylated regions (MeDiP DNA) and non-hypermethylated regions (input DNA) of female blood and placenta samples were cohybridized on a high resolution oligo-array. The microarray platform was a high resolution oligo-array of chromosomes 13, 18, 21, X and Y. Differentially methylated regions were identified using the SW-ARRAY algorithm. [33] As a result, more than 10,000 candidate DMRs were isolated.[32] This study provided numerous DMRs that could be potentially used for NIPD of aneuploidies of chromosomes 13, 18, 21, X and Y. The MeDIP and real time qPCR based approach[32,34], was developed and validated using 80 samples of maternal peripheral blood, from 46 women bearing a normal fetus and 34 bearing a Down syndrome fetus, and provided 100% specificity and 100% sensitivity.[34]

Although methods used in NIPD have high specificity and sensitivity yet it is not feasible considering the method ready for implementation in clinical practice due to lack of technical expertise in these methods.

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